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ORIGINAL

# Apoptotic and necrotic basal forebrain cholinergic neuronal loss and dendritic spines alteration after acute and long-term chlorpyrifos exposure: Legal implications of the use of toxicogenomic profile as a biomarker of harmful effects induced under subclinical doses

*Muerte de neuronas colinérgicas de la región basal por necrosis y apoptosis, así como alteración de la densidad de espinas dendríticas tras la exposición aguda y a largo plazo a clorpirifos: Implicaciones legales del uso del perfil toxicogenómico como biomarcador de efectos dañinos inducidos a dosis subclínicas*

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## *Abstract*

***Introduction:*** Chlorpyrifos (CPF) is an organophosphate insecticide reported to induce both after acute and repeated exposure learning and memory dysfunctions, although the mechanism is not completely known. CPF produces basal forebrain cholinergic neuronal loss, involved on learning and memory regulation, which could be the cause of such cognitive disorders. This effect was reported to be mediated through apoptotic process, although neuronal necrosis was also described after CPF exposure. Otherwise, neuronal dendritic spines were reported to be also involved on learning and memory process regulation and their alteration could also contribute to this effect. In this regard, CPF has been reported to induce an alteration in the dendritic spines density in the prefrontal cortex and hippocampus after acute and repeated exposure to subclinical doses respectively, thus their alteration in basal forebrain cholinergic neurons could also mediate cognitive disorders.

***Objectives and methods:*** Accordingly, we hypothesized that CPF induces basal forebrain cholinergic dendritic spine alteration at low concentrations and at higher concentrations produces necrotic and apoptotic cell death. We evaluated in septal SN56 basal forebrain cholinergic neurons, the CPF effect after 24 h and 14 days exposure on dendritic spines, the necrosis induction and the apoptotic and necrotic gene expression pathways.

***Results:*** This study shows that CPF induces after acute and long-term exposure an alteration of dendritic spines at lower concentrations than which induces cell death. Evaluation of cell death pathways and genes related to dendritic spine plasticity revealed that some of them are altered at lower concentrations than which produces the effects observed and below the No Observed Adverse Effect (NOAEL).

***Conclusions:*** The present finding suggest that the use of gene expression profile could be a more sensitive and accurate way to determine the NOAEL.

*Keywords:* Basal forebrain cholinergic neurons, chlorpyrifos, necrosis, apoptosis, dendritic spines, LOATEL, health legislation

## Resumen

***Introducción:*** El clorpirifos (CPF) es un insecticida organofosforado que tras la exposición aguda y repetida, induce disfunciones de los procesos de aprendizaje y memoria, aunque el mecanismo por el cual se produce este efecto no se conoce por completo. El CPF produce en la región cerebral basal anterior la pérdida de neuronas colinérgicas, que participan en la regulación de los procesos de aprendizaje y la memoria, pudiendo ser esta la causa de tales trastornos cognitivos. Se ha observado que este efecto está mediado a través del proceso de apoptosis, aunque también se ha descrito que se produce necrosis neuronal tras la exposición a CPF. Por otra parte, también se ha demostrado que las espinas dendríticas participan en la regulación de los procesos de aprendizaje y memoria y su disrupción también podría contribuir a la alteración de dichos procesos. En este sentido, se ha descrito que el CPF altera la densidad de las espinas dendríticas en la corteza prefrontal y el hipocampo tras la exposición aguda y repetida a dosis subclínicas, respectivamente, por lo que su perturbación en las neuronas colinérgicas de la región basal anterior también podría mediar estos trastornos cognitivos.

***Objetivos y métodos:*** De acuerdo con lo expuesto, nosotros hipotetizamos que el CPF induce, en las neuronas colinérgicas de la región basal anterior, una alteración de las espinas dendríticas a bajas concentraciones y a concentraciones más altas produce muerte celular por apoptosis y necrosis. Evaluamos en neuronas colinérgicas SN56 de la región basal anterior, el efecto del CPF

después de 24 horas y 14 días de exposición sobre las espinas dendríticas, la inducción de necrosis y las vías de expresión génica que median la inducción de apoptosis y necrosis.

***Resultados:*** Este estudio demuestra que el CPF induce, tras la exposición aguda y a largo plazo, una alteración de las espinas dendríticas, a concentraciones más bajas de aquellas a las que induce la muerte celular. La evaluación de las vías de muerte celular y los genes relacionados con la plasticidad de la espina dendrítica reveló que algunos de estos genes están alterados a concentraciones más bajas de aquellas a las que producen muerte celular o alteración de las espinas dendríticas y por debajo del Nivel sin efecto adverso observable (NOAEL) .

***Conclusiones:*** El presente studio sugiere que el uso del perfil de expresión génica podría ser una manera más sensible y precisa para la determinación del NOAEL.

*Palabras clave:* Neuronas colinérgicas de la region basal anterior basal, clorpirifos, necrosis, apoptosis, espinas dendríticas, LOATEL, regulación sanitaria

## Introduction

Chlorpyrifos (CPF) is an organophosphate (OP) insecticide widely used in domestic, agricultural, and industrial applications[1]. Human epidemiological studies have related OPs occupational exposure with neurological and neuro-behavioral deficits including impairments of learning and memory process[2,3]. In this regard, CPF has been shown to produce learning deficits in rats, after acute and repeated administration[4-6]. It has been suggested that inhibition of cholinesterase activity by CPF could be involved in these effects[7]. However, human studies of occupational exposure to OPs often fail to find a significant correlation between blood cholinesterase activity and neuro-behavioral deficits[2,3].

Otherwise, degeneration of septal cholinergic neurons that project to hippocampus has been linked to memory deficits that result from cholinergic modulation of hippocampal synaptic circuit loss[8]. Degeneration of septo-hippocampal cholinergic neurons, as seen in AD and other neurodegenerative diseases, results in loss of cholinergic modulation of septo-hippocampal synaptic circuits that leads to memory deficits[8]. In fact, the severity of memory deficit is strongly correlated with the degree of cholinergic cell loss[9]. Thus, cholinergic neuronal loss in this region could be related with CPF impairment of memory function among other actions[10].

In this regard, Lopez-Granero et al[4] reported that chronic dietary exposure in rats produced cognitive and emotional disorders related with changes in AChE forms. In addition, Del Pino et al[11] reported that CPF induced, after acute and long-term exposure, apoptotic cell death in cholinergic neurons from the basal forebrain and this effect was independent of AChE inhibition and acetylcholine level alteration, but was mediated partially by AChE-R and AChE-S overexpression, supporting the idea that the cognitive disorders reported after CPF exposures may be produced by induction of basal forebrain cholinergic cell loss mediated partially through AChE forms alteration. In addition, CPF has also been reported to induce neuronal cell death through necrosis process after CPF acute and long-term exposure[12,13], thus the cell death observed could be also mediated through necrosis induction by CPF. In this regard, previously Del Pino et al[14] reported that AChE-R and AChE-S overexpression induces at low concentrations apoptosis and necrosis at high concentrations in basal forebrain cholinergic neurons, supporting our hypothesis.

Otherwise, loss of dendritic spines has been reported in neurodegenerative disorders as AD affecting mostly selective neuronal networks of critical importance for memory and cognition, such as the basal forebrain cholinergic system, the medial temporal regions, the hippocampus and many neocortical association areas[15] inducing cognitive disorders[16,17]. In this regard, CPF has been reported to induce an alteration in the dendritic spines density in the prefrontal cortex and hippocampus after acute and repeated exposure to subclinical doses respectively[18,19]. Moreover, cognitive disorder has been reported to be produce from subclinical doses and from a lower doses from which basal forebrain cholinergic cell loss has been reported[20], as happens with dendritic spines alteration.

According to these data, we hypothesized that CPF induces basal forebrain cholinergic dendritic spine alteration at low concentrations and at higher concentrations produces neuronal loss through necrosis and apoptosis. The present work intends to study the CPF mechanisms of basal forebrain cholinergic neuronal loss and dendritic spine alteration, due to the importance of these effects to explain CPF toxicity on cognitive disorders and neurodegenerative diseases symptoms like. To reach this aim we treated with CPF for 24 h or repeatedly for 14 days, wild type or transfected with siRNA for AChE, SN56 cells from basal forebrain as an *in vitro* model of cholinergic neuronal cells from this region to research the CPF effect on dendritic spines and expression of genes involved in dendritic outgrowth, the necrotic cell death induction through AChE splice variants alteration and the apoptotic and necrotic gene expression pathways related to cell death process.

## Materials and methods

### *Chemicals*

The compounds chlorpyrifos (99.99%), poly-L-lysine, dibutyryl-cAMP, retinoic acid, 3,3´-diaminobenzidinetetrachloride, anti-microtubule-associated protein-2 (MAP-2) monoclonal antibody and paraformaldehyde were obtai-

ned from Sigma (Madrid, Spain). All other chemicals were reagent grade of the highest laboratory purity available.

### *Culture of SN56 cells*

SN56 cells, a cholinergic murine neuroblastoma cell line derived from septal neurons[21], were used as a model of cholinergic neurons from basal forebrain to evaluate CPF toxic effects on this specific type of neurons and the mechanisms through which they are induced. The cells were maintained at 37ºC and 5% $CO_2$ in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), penicillin/streptomycin, 2 mM L-glutamine (Sigma, Madrid, Spain), and 1 mM sodium pyruvate. Medium was changed every 48 h[22]. Differentiation of the cells was achieved by culturing for 3 days with 1 mM dibutyryl-cAMP and 1 µM retinoic acid as described[23,24], which produce morphological maturation and 3-4-fold increase of ChAT activity and acetylcholine level in the cells. Differentiated cells have been reported to be more sensitive to neurotoxic compound that affects cholinergic pathways[23,24].

In order to determine the dendritic spine density, the cellular lactate dehydrogenate (LDH) content in wild type and siRNA AChE transfected cells, the apoptotic and necrotic main gene expression pathways, and the expression of the main genes related with dendritic spines plasticity, cells were seeded in 6-well plates at a density of $10^6$ cells/well. Cells were treated for 24 h or for 14 days with CPF in concentrations between 0.01 µM to 70 µM and 0.01 µM to 40 µM respectively. At least 3 replicate wells/treatment were used. A vehicle group was employed in parallel for each experiment as a control.

In the literature, 10–100 µM chlorpyrifos has been routinely used to study chlorpyrifos toxicity[25-28], although there are not enough data regarding the relative distribution or concentration of CPF in human brain after acute and chronic exposure. In addition, studies have shown that the blood plasma concentration of CPF from human volunteers were similar to 0.1 µM[29]. Moreover, whole-body molar concentrations associated with the doses of CPF (2.5–25.0 mg/kg/day) used in behavioral experiments have been reported to be calculated as ranging between approximately 7.0 and 8.0 to 70.0 and 80.0 µM[20]. The NOAEL set for CPF is 0.1 mg/kg bw per day[30], which would be around 0.3 µM concentration in the tissues according to Terry et al[20]. The used doses appear to be relevant to study the cognitive disorders according to all described above. Furthermore, we chose CPF 30 µM concentration, which was the lowest concentration observed to induce cell death after acute exposure[11], to study the CPF necrotic and apoptotic mechanisms.

### *Lactate dehydrogenase (LDH) assay*

The necrotic cell death was assessed by measuring the LDH released into the culture medium using Lactate Dehydrogenase Activity Assay Kit (Sigma-Aldrich, Madrid, Spain) according to the manufacturer's instructions. Briefly, the culture medium was removed and pipetted into 96-well plates. The Master Mix reagent was added, and after 3 min colorimetric intensity was determined at 450 nm over every 5 min using a microplate spectrophotometer (Fluoroskan Ascent FL Microplate Fluorometer and Luminometer, ThermoFisher Scientific, Madrid, Spain).

### *Gene knockdown*

SN56 cells were transfected with siRNAs in 6-well plates (1 x$10^6$ cells/well) using HiPerfect Transfection reagent according to the manufacturer's instructions (Qiagen, Barcelona, Spain). Two sets of siRNA duplexes (Qiagen, Barcelona, Spain) homologous to mouse AChE sequence were designed using the Hi-Performance Design Algorithm (Norvatis AG) and were purchased from Qiagen (catalog numbers GS11423 a). As a transfection control, an All Stars Negative Control siRNA (Qiagen, Barcelona, Spain) was used. 48 h after transfection, the efficiency of siRNA-mediated AChE was determined by RT-PCR using primers specific for mouse AChE mRNA (Qiagen, Barcelona, Spain). The effects of AChE knockdown on cell injury were tested by LDH cell viability assay. After 24 h of incubation with the siRNAs, the cells were washed with PBS and incubated for a further 24 h or 14 days in culture medium with or without CPF.

### *Protein determination*

At the end of the treatments, SN56 cells were washed with pre-chilled PBS, collected by scrapping, and lysed using RIPA buffer (Thermo Scientific, Madrid, Spain) with freshly added protease inhibitors cocktail (ThermoFisher Scientific, Madrid, Spain). After centrifugation at 10,000 x g for 10 min at 4ºC, cell lysate supernatant was collected. Protein concentration was assayed using a BCA kit (ThermoFisher Scientific, Madrid, Spain) and normalized.

### *Dendritic spines density determination.*

SN56 differentiated cell were suspended in DEMEM medium. Cells suspension was plated at a density of 106 cell/ml onto poly-L-lysine 0.10 mg/mL coated glass coverslips (22 mm diameter) placed in plastic Petri dishes (35 mm diameter) coated with poly-L-lysine. Coverslips with adhered SN56 cells were fixed with ice-cold 4% (w/v) paraformaldehyde for 15 min and then placed into 0.5 M potassium/PBS (KPBS) until use. Coverslips were washed three times in KPBS, and cells were permeabilized with 50% ethanol for 1 h and blocked with 10% normal goat serum in 0.1% Triton X-100-supplemented KPBS (0.1% KPBST). After three 15 min KPBS washes, coverslipped cells were then incubated overnight in primary anti-microtubule-associated protein-2 (MAP-2) monoclonal antibody at 1:1000 dilution in 0.1% KPBST at 4°C. Three washes of 0.1% KPBST was then followed by immersion for 2 h in secondary anti-mouse antibody conjugated to biotin (Vector Laboratories) diluted 1:500 in 0.1% KPBST, washed in 0.1% KPBST, and visualized using Vectastain-avidin–biotin horseradish peroxidase complex (Vector

Laboratories, Barcelona, Spain) at 1:1000 dilution in 0.1% KPBST for 1 h before staining with 0.05% (w/v) 3,3´-diaminobenzidinetetrachloride, 0.005% (v/v) $H_2O_2$, and 2.5% (w/v) nickel (II)sulfate in 0.175 M sodium acetate[31]. MAP-2 protein is not localized in dendritic spines but allows for accurate quantification of dendritic spine density.

### *Real-time PCR analysis*

Total RNA was extracted using the Trizol Reagent method (Invitrogen, Madrid, Spain). The final RNA concentration was determined using a spectrophotometer Nanodrop 2000 (ThermoFisher Scientific, Madrid, Spain) and the quality of total RNA samples was assessed using an ExperionLabChip (Bio-Rad, Madrid, Spain) gel. First-strand cDNA was synthesized with 1000 ng of cRNA by using a PCR array first strand-synthesis kit (C-02; SuperArray-Bioscience, Madrid, Spain) following the manufacturer's instructions and including a genomic DNA elimination step and external RNA controls. After reverse transcription, we performed QPCR using the mouse Cell Death PathwayFinder PCR Array (PAMM-212Z) used to analyze mRNA levels of 84 key genes involved in cell death, in a 96-well format, according to the manufacturer's instructions (SABiosciencesInc). Reactions were run on a CFX96 using Real-Time SYBR Green PCR master mix PA-012 (SABiosciencesInc). The thermocycler parameters were 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Relative changes in gene expression were calculated via SuperArray PCR Array Data Analysis Software using the cycle threshold (Ct) method with normalization of the raw data to several housekeeping genes. The expression data are presented as actual change multiples. We also performed QPCR using prevalidated primer sets (SuperArray Bioscience) for mRNAs encoding Postsynaptic Density Protein 95 (PSD95; PPH01848A), Spinophilin (SPN; PPM34114A), Synaptophysin (SYP; PPM03241A), N-Methyl-D-Aspartate Receptor Subunit NR1 (NMDAR1;PPH01823F), AChE (PPM35356A), and ACTB (PPM02945B). We used ACTB as an internal control for normalization. Reactions were run on a CFX96 using Real-Time SYBR Green PCR master mix PA-012 (SuperArray Bioscience). The thermocycler parameters were 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 72°C for 30 seconds. Relative changes in gene expression were calculated using the Ct (cycle threshold) method. The expression data are presented as actual change multiples32 (Livak and Schmittgen 2001).

We chose PSD95, NMDAR1, SPN and SYP genes to determine the effect of CPF on dendritic spine density, because both genes are the main regulators of dendritic spine plasticity[33,35].

### *Statistical analysis*

At least three replicates for each experimental condition were performed, and the presented results were representative of these replicates. Data are represented as means ± standard deviation (SD). Comparisons between experimental and control groups were performed by Student's *t* test and ANOVA analyses followed by the Tukey post-hoc test. Statistical difference was accepted when $p \leq 0.05$. Statistical analysis of data was carried out by computer using GraphPad software.

## Results

### *Effect of CPF on SN56 cell viability*

We used the LDH assay to evaluate cell survival after 24 h and 14 days exposure to CPF at increasing concentrations. LDH is released from cells as a result of loss of plasma membrane integrity and it is indicative of necrotic cell death mechanism. An increased LDH release to the medium was observed in a dose dependent way after 24 h and long-term CPF exposure from 50 µM and 20 µM concentrations respectively compared with vehicle-treated cells (control negative) (**Figure 1**). Moreover, after CPF treatment of SN56 AChE silenced cells amelioration in LDH release was also observed (**Figure 1**). There was no significant difference between data of vehicle-treated cells and control cells.

### *AChE gene knockdown*

To investigate the protection afforded by the knockdown of the AChE during cell death, we introduced siRNA oligonucleotides into SN56 cells by transfection directed against AChE. Transfection of cells with control siRNA showed no effect on AChE gene expression or cell viability, but the AChE siRNA caused large reductions in AChE gene expression (**Figure 2**). Cultures transfected with control siRNA compared with culture transfected against AChE, showed no effect in cell viability by LDH assays (**Figure 2**).

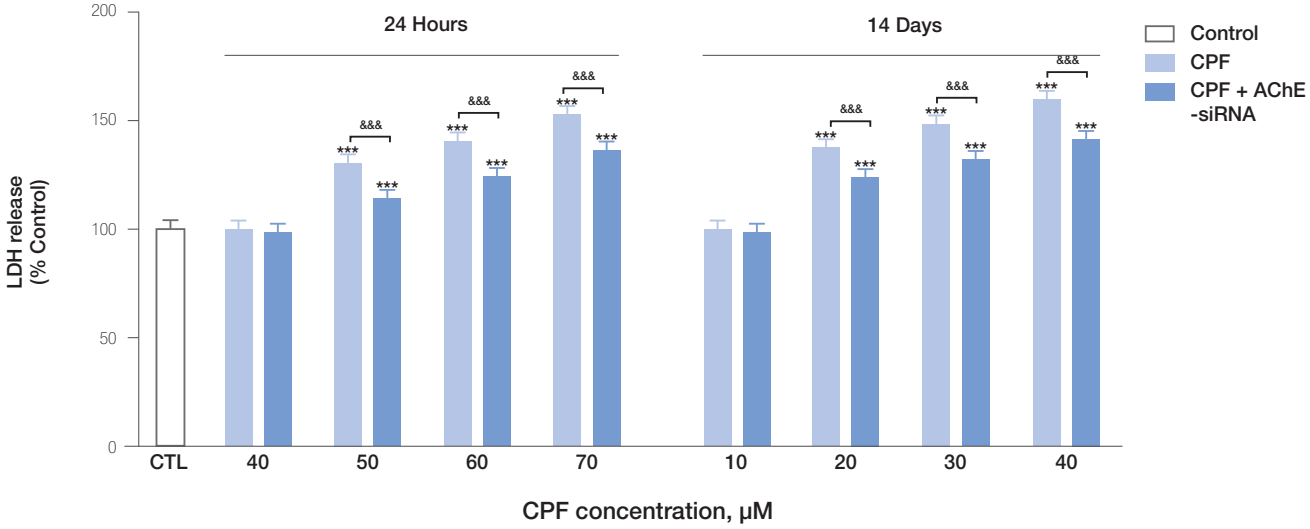
### *Effect of CPF on dendritic spine density*

We observed that CPF induces acute (from 1 µM) and long-term (from 0.1 µM), concentration-dependent, reduction in the number of dendritic spine-like processes per length of neurite compared with vehicle treated on SN56 cultured neurons (**Figure 3**). There was no effect of treatment on mean soma size or the number of neurites per neuron (Data not shown).

### *Real-time PCR analysis*

After incubation for 24 h and 14 days with 0.01 µM and 30 µM concentrations of CPF in SN56 cells, necrotic and apoptotic gene expression profile of CPF was analyzed with our Real-time PCR arrays. Moreover, the CPF effect on AChE and the main regulator dendritic spines synaptic plasticity gene expression in AChE silenced Cells and wild type respectively were analyzed. The gene expression profile of CPF was significantly different from normal control. The results show that after 24 h and 14 days exposure at 0.01 µM concentration of CPF only the expression of TNF, TNFRSF1A, TNFRSF10A, TNFRSF11B proapoptotic

**Figure 1:** CPF (0.01 to 70 µM) effect on cell viability of SN56 wild type or AChE silenced was determined by LDH release assays. Results are expressed as percentages of LDH release after subtracting the control values. Data represents the mean ± SD of three separate experiments from cells of different cultures, each one performed in triplicate. ***p<0.001 compared to control. &&&p≤0.001 compared to CPF treatment.




genes, TRAF2, BICR2 and BIRC3 antiapoptotic genes and MAG, S100A7A and KCN1P1 genes related with necrosis process was induced (**Table I** and **II**). Moreover, after 24 h exposure at 30 µM concentration of CPF 27 genes were up-regulated and 5 genes were down-regulated with a fold change higher and lower than 1.5 respectively for CPF compared with normal control (**Table I**). In addition, after and 14 days exposure at 30 µM concentration of CPF 43 genes were up-regulated and 7 genes were down-regulated with a fold change higher and lower than 1.5 respectively for CPF compared with normal control (**Table II**). Finally, we observed that after acute (from 0.01 µM) and long term (from 0.01 µM) CPF exposure, a concentration-dependent reduction in PSD95 and SYP gene expression (**Figure 4A** and **4B**), but we only observed a concentration-dependent gene expression reduction of SPN and NMDANR1 after acute exposure from 1 µM CPF concentration and after long-term exposure from 0.1 µM CPF concentrations (**Figure 4C** and **4D**).

## Discussion

In the present work we show that CPF induces acute (from 50µM) and long-term (from 20 µM), concentration-dependent, necrotic cell death on septal SN56 cholinergic basal forebrain neurons. These results are supported by previous works which showed that CPF induced neuronal cell death through necrosis process after CPF acute and long-term exposure[12,13]. Previously, we found that CPF induces acute (from 30µM) and long-term (from 1 µM), concentration-dependent, cell death on septal SN56 cholinergic basal forebrain neurons, showing that apoptotic process is involved in this effect mediated partially by AChE variants overexpression[11]. Moreover, we show that the increase in LDH release was mediated partially by AChE overexpression. In this regard, Del Pino et al[14] reported that AChE-R and AChE-S overexpression induces at low concentrations apoptosis and necrosis at higher concentrations in basal forebrain cholinergic neurons, supporting our results. Basal forebrain cholinergic neurons loss has been related with cognitive deficits[36-40]. In fact, the severity of memory deficit is strongly corre-

**Figure 2:** Downregulation of AChE in SN56 cells and its impact on cell viability and gene expression was determined. Control: SN56 cells transfected without siRNA. Negative (Neg.) control: SN56 cells transfected with scrambled siRNA. AChE-siRNA: transfected with siRNA against AChE. (A) LDH assays shows that AChE downregulation did not significantly induce cell damage after 48 h. (B) AChE downregulation could be detected by RT-PCR analysis 48 h after transfection. Values are given as mean ± SD of three separate experiments from cells of different cultures, each one performed in triplicate. ***p<0.001 compared to control.

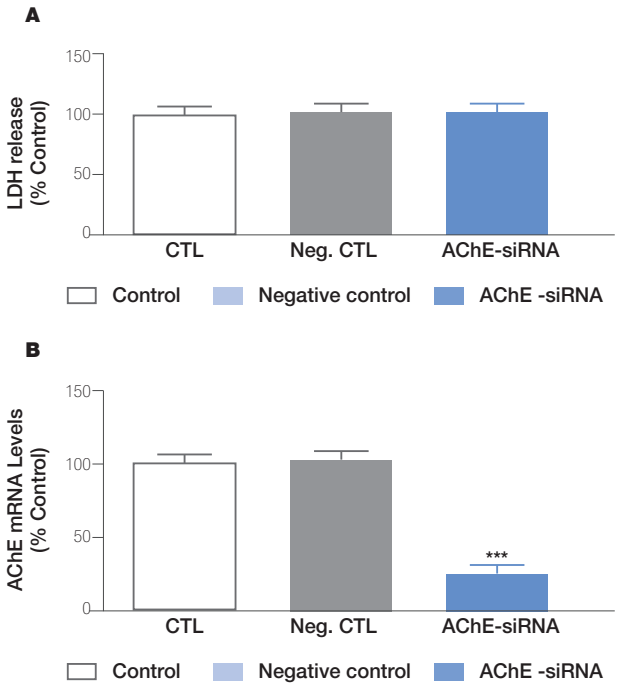


**Figure 3:** Effects of different CPF concentrations for 24 h or 14 days on dendritic spine density were determined in neurite SN56 cells. Dendritic spine density is indicated as % of control (CTL, white column). Data represents the mean ± SD of three independent experiments in triplicate. ***p<0.001 compared to control.

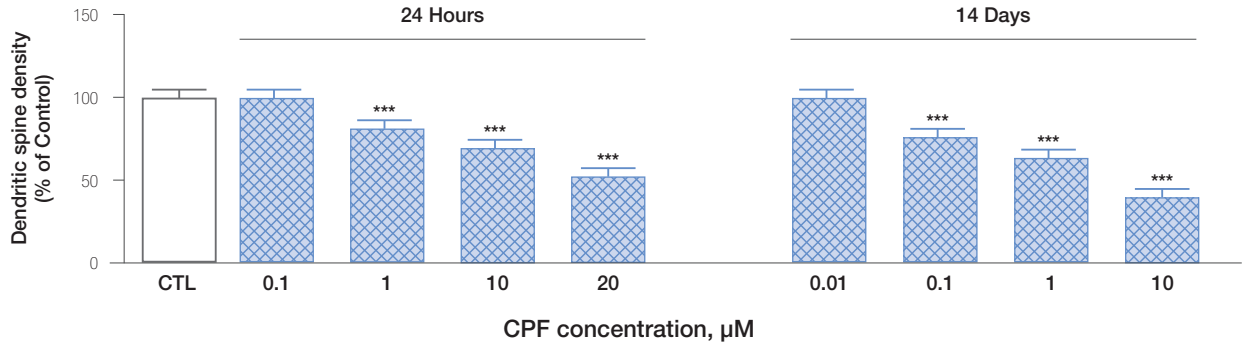



**Figure 4:** Shows results from real-time PCR arrays targeting (A) PSD95, (B) SYP, (B) SPN and (D) NMDAR1 genes after 24 h and 14 days CPF treatment. SD95, SYP, SPN and NMDAR1 gene expression was compared with controls. Each bar represents mean ± SD of 6 samples. Levels were measured using QPCR. ACTB was used as an internal control. ***p<0.001, significantly different from controls.

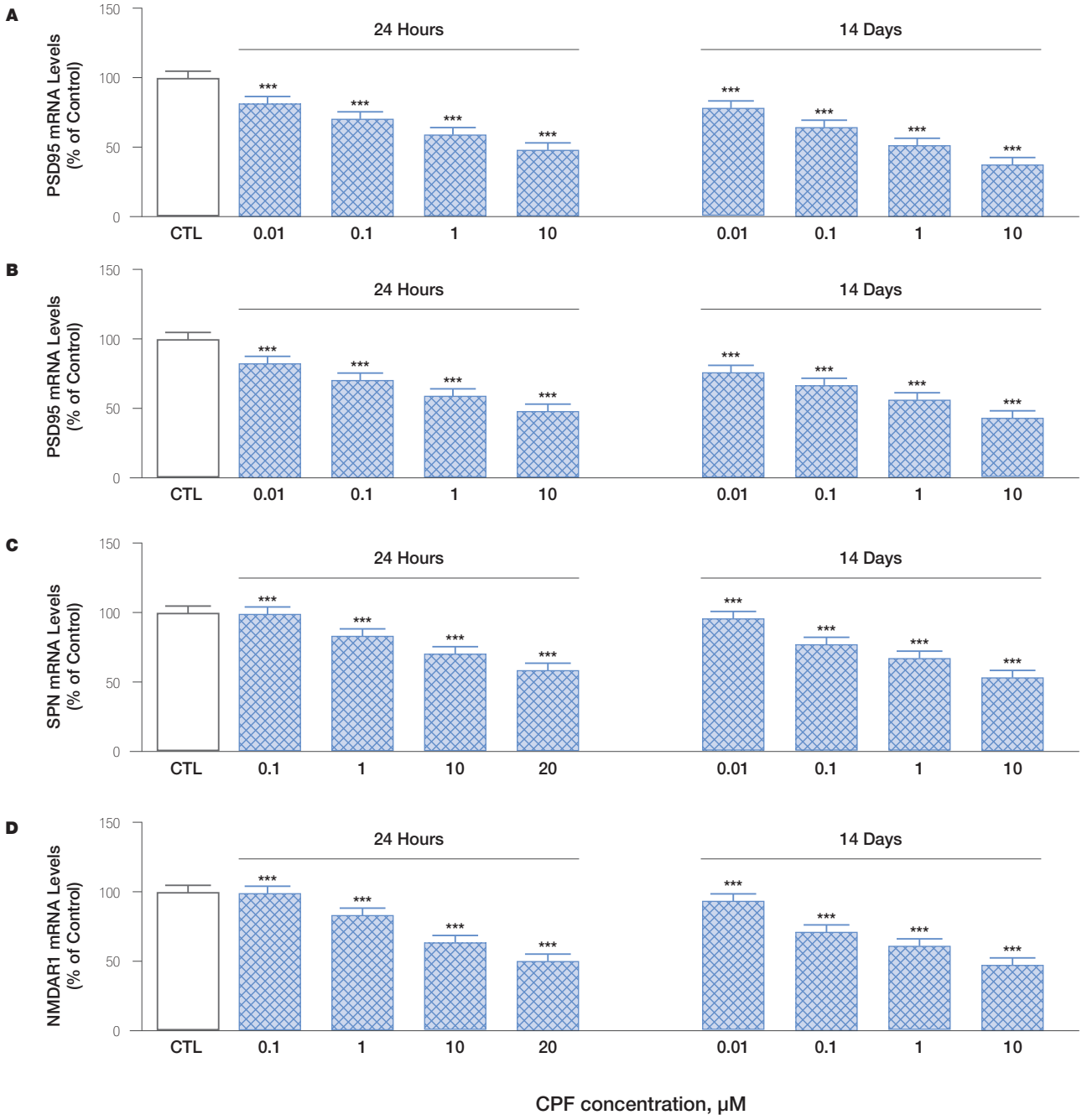


**Table I:** Fold-change of cell death related genes after 24 h CPF treatment. Values on red or blue mean a fold change upper or downer than 2

| Gene Symbol | GenBank Accession No. | Fold Change | | Gene Symbol | GenBank Accession No. | Fold Change | |
|---|---|---|---|---|---|---|---|
| | | 0.01μM | 30μM | | | 0.01μM | 30μM |
| ABL1 | NM_005157 | 1,11[b] | 1,23[a] | FOXI1 | NM_012188 | 1,12[a] | 0,07 |
| AKT1 | NM_005163 | 1,02 | 1,05 | GAA | NM_000152 | 1,09 | 1,19[a] |
| APAF1 | NM_001160 | 1,15[b] | 2,52[a] | GADD45A | NM_001924 | 1,11[a] | 2,24[a] |
| APP | NM_000484 | 1,16[a] | 1,41[a] | GALNT5 | NM_014568 | 1,03 | 1.05 |
| ATG12 | NM_004707 | 1,17[a] | 1,13[a] | GRB2 | NM_002086 | 1,06 | 1,09 |
| ATG16L1 | NM_017974 | 1,21[a] | 1,16[a] | HSPBAP1 | NM_024610 | 1,05 | 1,13[a] |
| ATG3 | NM_022488 | 1,04 | 1,11[a] | HTT | NM_002111 | 1,06 | 1,23[a] |
| ATG5 | NM_004849 | 1,01 | 1,21[a] | IFNG | NM_000619 | 1,07 | 1,08 |
| ATG7 | NM_006395 | 1,04 | 1,04 | IGF1 | NM_000618 | 1,01 | 1,14[a] |
| ATP6V1G2 | NM_130463 | 1,02 | 1,02 | IGF1R | NM_000875 | 1,02 | 1,09 |
| BAX | NM_004324 | 1,05 | 2,54[a] | INS | NM_000207 | 1,07 | 1,11[b] |
| BCL2 | NM_000633 | 1,10[b] | 1,15[a] | IRGM | NM_001145805 | 1,13[b] | 1,28[a] |
| BCL2A1 | NM_004049 | 1,02 | 1,04 | JPH3 | NM_020655 | 1,09 | 1,06 |
| BCL2L1 | NM_138578 | 1,13[a] | 2,23[a] | KCNIP1 | NM_014592 | 2,51[a] | 3,21[a] |
| BCL2L11 | NM_006538 | 1,01 | 2,32[a] | MAG | NM_002361 | 2,12[a] | 2,69[a] |
| BECN1 | NM_003766 | 1,03 | 1,06 | MAP1LC3A | NM_181509 | 1,08 | 2,35[a] |
| BIRC2 | NM_001166 | 1,72[a] | -1,43[a] | MAPK8 | NM_002750 | 1,07 | 1,17[b] |
| BIRC3 | NM_001165 | 1,69[a] | -1,59[a] | MCL1 | NM_021960 | 1,14[a] | 1,27[a] |
| BMF | NM_033503 | 1,06 | 1,06 | NFKB1 | NM_003998 | 1,02 | 1,06 |
| C1orf159 | NM_017891 | 1,13[a] | 1,16[a] | NOL3 | NM_003946 | 1,05 | 1,04 |
| CASP1 | NM_033292 | 1,05 | 2,21[a] | OR10J3 | NM_001004467 | 1,01 | 1,07 |
| CASP2 | NM_032982 | 1,01 | 1,09 | PARP1 | NM_001618 | 1,11[a] | 1,03 |
| CASP3 | NM_004346 | 1,05 | 1,57[a] | PARP2 | NM_005484 | 1,06 | 1,01 |
| CASP6 | NM_032992 | 1,06 | 1,25[a] | PIK3C3 | NM_002647 | 1.07 | 1.05 |
| CASP7 | NM_001227 | 1,02 | 2,31[a] | PVR | NM_006505 | 1,06 | 1,10[a] |
| CASP9 | NM_001229 | 1,01 | 1,69[a] | RAB25 | NM_020387 | 1,03 | 1,02 |
| CCDC103 | NM_213607 | 1,07 | 1,05 | RPS6KB1 | NM_003161 | 1,27[a] | 1,31[a] |
| CD40 | NM_001250 | 1,08 | 3,31[a] | S100A7A | NM_176823 | 2,17[a] | 2,47[a] |
| CD40LG | NM_000074 | 1,06 | 3,56[a] | SNCA | NM_000345 | 1,06 | 1,25[a] |
| CFLAR | NM_003879 | 1,21[a] | 1,11[a] | SPATA2 | NM_006038 | 1,12[a] | 2,41[a] |
| COMMD4 | NM_017828 | 1,02 | 1,02 | SQSTM1 | NM_003900 | 1,17[a] | 1,12[a] |
| CTSB | NM_001908 | 1,13[a] | 1,05 | SYCP2 | NM_014258 | 1,03 | 1,66[a] |
| CTSS | NM_004079 | 1,08 | 1,04 | TMEM57 | NM_018202 | 1,11[a] | 1,15[a] |
| CYLD | NM_015247 | 1,12[a] | 1,08 | TNF | NM_000594 | 1,53[a] | 1,69[a] |
| DEFB1 | NM_005218 | 1,01 | 1,01 | TNFRSF10A | NM_003844 | 1,61[a] | 1,78[a] |
| DENND4A | NM_005848 | 1,03 | 1,33[a] | TNFRSF11B | NM_002546 | 2,01[b] | 2,37[b] |
| DFFA | NM_004401 | 1,05 | 1,09 | TNFRSF1A | NM_001065 | 1,75[a] | 1,91[a] |
| DPYSL4 | NM_006426 | 1,01 | 1,05 | TP53 | NM_000546 | 1,10 | 1,67[a] |
| EIF5B | NM_015904 | 1,03 | 1,33[b] | TRAF2 | NM_021138 | 2,22[a] | 2,72[a] |
| ESR1 | NM_000125 | 1,04 | 1,07 | TXNL4B | NM_017853 | 1,01 | 1,69[a] |
| FAS | NM_000043 | 1,02 | 2,31[a] | ULK1 | NM_003565 | 1,02 | 1,04 |
| FASLG | NM_000639 | 1,06 | 2,13[a] | XIAP | NM_001167 | 1,05 | 1,01 |

[a] $p < 0.001$ compared to the control. [b] $p < 0.05$ compared to the control.

lated with the degree of cholinergic cell loss[9]. Thus, the observed effects on cholinergic basal forebrain neurons may be, in part, responsible for the learning deficits and neurodegenerative disease symptoms like observed after acute and long-term exposure to CPF.

Moreover, after acute (from 0.01 μM) and long-term (from 0.01 μM) CPF exposure we only observed an induction in the gene expression of TNF, TNFRSF1A, TNFRSF10A, TNFRSF11B proapoptotic genes, TRAF2, BICR2 and BIRC3 antiapoptotic genes and MAG, S100A7A and KCN1P1 genes related with necrosis process. Apoptosis is triggered through the extrinsic pathway when extracellular stimuli and the cytokines CD40L, FASLG, TNFSF10 and TNF-α inducing cytokine receptors, including FAS receptor and tumor necrosis factor super family receptors (TNFRs) like TNFRSF1A, TNFRSF1B, TNFRSF10B and CD40, are activated[41,42]. Inhibitors of apoptosis BIRC3 and BIRC2, through TRAF2, can prevent its activation[43]. According to this, CPF could activate cell death through extrinsic partway, but was blocked by activation of apoptosis inhibitors. Otherwise, MAG and S100A7A genes have been related with necrosis induction[44] and KChIP1 expression has been reported to play a protective role against cell death and its downregulation leads to the induction of necrotic cell death[45]. Therefore, KChIP1 overexpression after CPF exposure could be a protective mechanism against cell injury produced by CPF.

In addition, after acute (30 μM) CPF exposure the gene expression resultant showed a fold change greater or lower than 1.5 in the expression of the 29 genes related to apoptosis and necrosis for CPF treatment. Our research showed that CPF increased the expression of CD40, CD40LG, FAS, FASLG, TNF, TNFRSF10A, TNFRSF1A, TNFRSF11B, which likely leaded to induce

**Table II:** Fold-change of cell death related genes after 14 days CPF treatment. Values on red or blue mean a fold change upper or downer than 2

| Gene Symbol | GenBank Accession No. | Fold Change | | Gene Symbol | GenBank Accession No. | Fold Change | |
|---|---|---|---|---|---|---|---|
| | | 0.01µM | 30µM | | | 0.01µM | 30µM |
| ABL1 | NM_005157 | 1,15[b] | 1,32[a] | FOXI1 | NM_012188 | 1,15[a] | 2,14[a] |
| AKT1 | NM_005163 | 1,07 | 1,08 | GAA | NM_000152 | 1,04 | 1,32[a] |
| APAF1 | NM_001160 | 1,18[a] | 2,56[a] | GADD45A | NM_001924 | 1,14[a] | 2,22[a] |
| APP | NM_000484 | 1,19[a] | 1,31[a] | GALNT5 | NM_014568 | 1,06 | -1,64 |
| ATG12 | NM_004707 | 1,18[a] | 1,24[a] | GRB2 | NM_002086 | 1,04 | 2,51[a] |
| ATG16L1 | NM_017974 | 1,19[a] | 1,33[a] | HSPBAP1 | NM_024610 | 1,07 | 1,69[a] |
| ATG3 | NM_022488 | 1,07 | 1,19[a] | HTT | NM_002111 | 1,03 | 1,13[a] |
| ATG5 | NM_004849 | 1,04 | 1,62[a] | IFNG | NM_000619 | 1,05 | 1,02 |
| ATG7 | NM_006395 | 1,03 | 1,84[a] | IGF1 | NM_000618 | 1,06 | 1,16[a] |
| ATP6V1G2 | NM_130463 | 1,01 | 1,59[a] | IGF1R | NM_000875 | 1,03 | 1,05 |
| BAX | NM_004324 | 1,09 | 2,69[a] | INS | NM_000207 | 1,06 | 1,11[a] |
| BCL2 | NM_000633 | 1,13[a] | 1,19[a] | IRGM | NM_001145805 | 1,10[b] | 1,29[a] |
| BCL2A1 | NM_004049 | 1,05 | 1,08 | JPH3 | NM_020655 | 1,05 | 2,38[a] |
| BCL2L1 | NM_138578 | 1,08 | 2,27[a] | KCNIP1 | NM_014592 | 2,62[a] | -3,67[a] |
| BCL2L11 | NM_006538 | 1,07 | 2,34[a] | MAG | NM_002361 | 2,25[a] | -3,42[a] |
| BECN1 | NM_003766 | 1,07 | 1,09 | MAP1LC3A | NM_181509 | 1,04 | 2,42[a] |
| BIRC2 | NM_001166 | 1,86[a] | -1,62[a] | MAPK8 | NM_002750 | 1,02 | 1,21[b] |
| BIRC3 | NM_001165 | 1,82[a] | -1,53[a] | MCL1 | NM_021960 | 1,10[b] | 1,29[a] |
| BMF | NM_033503 | 1,09 | 2,95[a] | NFKB1 | NM_003998 | 1,03 | 1,07 |
| C1orf159 | NM_017891 | 1,12[a] | 3,76[a] | NOL3 | NM_003946 | 1,06 | 1,02 |
| CASP1 | NM_033292 | 1,02 | 2,19[a] | OR10J3 | NM_001004467 | 1,05 | -3,67[a] |
| CASP2 | NM_032982 | 1,03 | 1,15[a] | PARP1 | NM_001618 | 1,13[a] | 1,74[a] |
| CASP3 | NM_004346 | 1,07 | 1,72[a] | PARP2 | NM_005484 | 1,02 | 1,81[a] |
| CASP6 | NM_032992 | 1,04 | 1,23[a] | PIK3C3 | NM_002647 | 1.05 | 2,28[a] |
| CASP7 | NM_001227 | 1,03 | 2,22[a] | PVR | NM_006505 | 1,08 | 1,49[a] |
| CASP9 | NM_001229 | 1,06 | 1,68[a] | RAB25 | NM_020387 | 1,04 | 1,04 |
| CCDC103 | NM_213607 | 1,13[a] | 2,31[a] | RPS6KB1 | NM_003161 | 1,15[a] | 1,19[a] |
| CD40 | NM_001250 | 1,08 | 4,48[a] | S100A7A | NM_176823 | 2,28[a] | -2,73[a] |
| CD40LG | NM_000074 | 1,06 | 3,16[a] | SNCA | NM_000345 | 1,03 | 1,29[a] |
| CFLAR | NM_003879 | 1,21[a] | 1,27[a] | SPATA2 | NM_006038 | 1,10[b] | 2,36[a] |
| COMMD4 | NM_017828 | 1,02 | 2,12[a] | SQSTM1 | NM_003900 | 1,12[a] | 1,19[a] |
| CTSB | NM_001908 | 1,13[a] | 2,52[a] | SYCP2 | NM_014258 | 1,05 | 1,67[a] |
| CTSS | NM_004079 | 1,05 | 2,26 | TMEM57 | NM_018202 | 1,13[a] | 1,24[a] |
| CYLD | NM_015247 | 1,10[b] | 2,22[a] | TNF | NM_000594 | 1,59[a] | 1,66[a] |
| DEFB1 | NM_005218 | 1,04 | 1,93[a] | TNFRSF10A | NM_003844 | 1,72[a] | 1,79[a] |
| DENND4A | NM_005848 | 1,01 | 1,35[b] | TNFRSF11B | NM_002546 | 2,14[b] | 2,26[b] |
| DFFA | NM_004401 | 1,03 | 1,03 | TNFRSF1A | NM_001065 | 1,83[a] | 1,91[a] |
| DPYSL4 | NM_006426 | 1,06 | 2,81 | TP53 | NM_000546 | 1,18 | 1,85[a] |
| EIF5B | NM_015904 | 1,01 | 1,33[b] | TRAF2 | NM_021138 | 2,31[a] | 3,14[a] |
| ESR1 | NM_000125 | 1,02 | 1,01 | TXNL4B | NM_017853 | 1,11 | 1,69[a] |
| FAS | NM_000043 | 1,05 | 2,36[a] | ULK1 | NM_003565 | 1,09 | 1,02 |
| FASLG | NM_000639 | 1,03 | 2,46[a] | XIAP | NM_001167 | 1,06 | 1,04 |

[a] $p < 0.001$ compared to the control. [b] $p < 0.05$ compared to the control.

apoptosis through extrinsic pathway. CPF increased also the expression of TRAF2 and decreased BIRC3 and BIRC2 antiapoptotic genes allowing caspases activation which expression was increased. Besides, CPF induced GADD45A, and TP53 genes expression. The P53-GADD45A pathway has been shown to primarily play a role in the control of G2-M arrest following certain DNA-damaging agents[46,47], thus their induction could lead to cell arrest. Moreover, CPF increased the expression of BAX and BCL2L11 proapoptotic genes and BCL2L1 antiapoptoptic gene[48], further decrease the ratio BCL2/BAX which regulated the induction of apoptosis[49] leading to apoptosis. Finally, CPF increased the expression of CASP1, CASP3, CASP7, CASP9 which executed apoptosis. Besides, CPF increased the expression of KCN1P1, MAG and S100A7A genes related to necrosis as happened at lower concentrations indicating that necrosis is prevented.

After long-term CPF exposure (30 µM), besides the effect on the same genes commented after acute exposure, CPF increased the expression of ATP6VG2, BMF, CLORF159, CCDC103, COMMD4, CTSB, STSS, CYLD, DEFB1, DPYSL4, FOXIL1, GRB2, HSPBAP1, JPH3, PARP1, PARP2, PIK3C3, PVR, TXNL4B and decreased the expression of GALNTL5, OR10J3, KCN1P1, MAG and S100A7A genes related to necrosis according with the research of Hitomi et al[44]. Our study indicates that CPF exerts its cell death effects by involving apoptosis and necrosis pathways.

Otherwise, we observed that CPF induces acute (from 1 µM) and long-term (from 0.1 µM), concentration-dependent, reduction in the number of dendritic spine density on septal SN56 cholinergic basal forebrain neurons. In this regard, CPF has been reported to induce an alteration in the dendritic spines density in the prefrontal cortex and hip-

pocampus after acute and repeated exposure to subclinical doses respectively[18,19], supporting our results. Dendritic spines loss has been reported in neurodegenerative disorders as AD, affecting mostly selective neuronal networks of critical importance for memory and cognition, such as the basal forebrain cholinergic system, the medial temporal regions, the hippocampus and many neocortical association areas[15] inducing cognitive disorders[16,17]. Moreover, cognitive disorders have been reported to be produced from subclinical doses and from lower doses from which basal forebrain cholinergic cell loss has been reported[20], as happens with dendritic spines alteration, suggesting that both processes are implicated in cognitive disorders induction, at low doses only through dendritic spine alteration and at higher doses through both mechanisms.

In addition, we observed that after acute (from 0.01 μM) and long term (from 0.01 μM) CPF exposure, a concentration-dependent downregulation in PSD95 and SYP gene expression was produced. It has been suggested that SYP plays a role in formation and stabilization of synapses[50], regulating dendritic spine density and shape[51,52]. Moreover, PSD95 was described to play a role in determining the size and the strength of synapses[53,54], formation of synapse assemblies[54] and spine-maturation[55,56]. Therefore, both genes alteration could be implicated in the effects observed on dendritic spines. Our results show that PSD95 and SYP gene expression is altered below the concentration from which dendritic spine density disruption is evident, which indicates that the harmful effects of CPF exposure on dendritic spines could be started and detected before they would be manifest and these genes could be used as biomarker of this effect. Moreover, PSD95 and SYP are both involved in dendritic spine morphology regulation and their alteration by CPF exposure may be involved with a disruption of dendrite spine shape, contributing to the cognitive disorders observed. Otherwise, we only observed a concentration-dependent SPN and NMDANR1 gene expression downregulation after acute exposure from 1 μM CPF concentration and after long-term exposure from 0.1 μM CPF concentrations as seen with dendritic spines density reduction, suggesting both genes are directly correlated with this effect. In this regard, SPN, a key cytoskeletal protein in the formation and maintenance of dendritic spines, has been reported to modulate dendritic morphology and number[33] and an increase in dendritic spine density has been correlated with an increase in the spinophilin expression[34]. In addition, N-methyl-D-aspartate (NMDA) receptor, is also involved in synaptic plasticity as well as learning and memory processes regulation[57] and the N-methyl-D-aspartate receptor antagonists reduce dendritic spine density and neurite growth[35]. Future studies are needed to determine the implication of these genes in dendritic spine shape and density disruption mechanisms.

According to all exposed above, CPF induced changes on cell death and dendritic spines plasticity pathways were detected at doses below those required to induce manifest apoptotic and necrotic cell death and alter dendritic spines plasticity. These results support the suggestion that gene expression changes could potentially be more sensitive measures of effects at early stages and at lower doses than many typical toxicological measures[58] and that altered toxicogenomic profiles contributing to toxicologically-relevant pathways provide useful tools for more precise determination of toxicological mechanisms as compared to traditional toxicological endpoints and for reducing uncertainty in establishing lowest observed adverse effect level (LOAEL), no observed adverse effect level (NOAEL), or benchmark dose (BMD)[59-62]. Therefore, the toxicogenomic profile analysis could offer means to improve human health risk assessment generally based on standard toxicity test results. Previously, this approach has been employed in different studies as the published by Lobenhofer et al[60] that examines *in vitro* transcriptional responses to very low concentrations of estrogen, confirming that estrogen concentrations below those which are physiologically relevant did not induce a measurable transcriptional response. That concentration threshold was referred to as the No Observable Transcriptional Effect Level (NOTEL). In our study, it was shown that the CPF Lowest Observed Adverse Transcriptional Expression Levels (LOATEL) for 12 responding genes was at least 100-fold lower than the NOAEL that was based on observable inhibition of erythrocyte acetylcholinesterase activity in humans[30]. Therefore, lower doses would be needed in order to observe no transcriptional effects. Examination of data from the group of 12 responding genes leads us to propose 0.003-0.004 mg/kg/day as LOATEL for CPF. The present study did not, therefore, establish a No Observed Adverse Transcriptional Effect Level (NOATEL) for CPF. These subclinical transcriptional changes may be employed as predictors of adverse effects from toxic compounds exposure and thereby used to improve risk assessment and safety evaluation. Further studies are required to confirm this LOATEL.

## Conclusion

Taking all together, it can be concluded that after acute and long-term exposure, CPF induces at low concentration a dendritic spine density reduction and at higher concentrations induces cell death on cholinergic neurons from basal forebrain through apoptotic and necrotic mechanisms, depending on the exposure concentration mediated in part though AChE variants overexpression and these effects were initiated at lower concentration that which they are manifest, as indicated by transcriptional alterations of key genes. Our results, particularly those for CPF treatment, suggest that toxicogenomic profiles provide a sensitive tool for identifying and characterizing thresholds of toxicity for potentially toxic compounds based upon a transcriptome-level of insight into their mechanisms. These effects could explain cognitive alterations and neurodegenerative diseases induced by CPF. In this regard, all the mechanisms that we reported to induce cholinergic cell loss in

basal forebrain have been described to be involved with the induction of cognitive disorders, which supports this hypothesis. Future studies should be developed to determine the other mechanisms implicated in these effects observed on cholinergic neurons. These results are of interest, since they provide new information on the mechanisms that mediate dendritic spine density alteration and cell death induce by CPF, and because they lead to a better understanding of some effects related to CPF toxicity and highlight the need for a new NOAEL and LOAEL and a new risk assessment of this pesticide.

## Conflicts of interest statement

There are no conflicts of interest to declare.

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